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BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Natural Killer Cells Ameliorate Liver Fibrosis by Killing Activated Stellate Cells in NKG2D-Dependent and Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Dependent Manners

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See editorial on page 600.

Background & Aims: Viral hepatitis infection, which is a major cause of liver fibrosis, is associated with activation of innate immunity. However, the role of innate immunity in liver fibrosis remains obscure. *Methods:* Liver fibrosis was induced either by feeding mice with the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet or by injecting them with carbon tetrachloride. The Toll-like receptor 3 ligand, polyinosinic-polycytidylic acid, was used to activate innate immunity cells and mediators, including natural killer cells and interferon γ. *Results:* In the mouse model of DDC-induced liver fibrosis, natural killer cell activation by polyinosinic-polycytidylic acid induced cell death to activated hepatic stellate cells and attenuated the severity of liver fibrosis. Polyinosinic-polycytidylic acid treatment also ameliorated liver fibrosis induced by carbon tetrachloride. The observed protective effect of polyinosinic-polycytidylic acid on liver fibrosis was diminished through either depletion of natural killer cells or by disruption of the interferon γ gene. Expression of retinoic acid early inducible 1, the NKG2D ligand, was undetectable on quiescent hepatic stellate cells, whereas high levels were found on activated hepatic stellate cells, which correlated with the resistance and susceptibility of quiescent hepatic stellate cells and activated hepatic stellate cells to natural killer cell lysis, respectively. Moreover, treatment with polyinosinic-polycytidylic acid or interferon γ enhanced the cytotoxicity of natural killer cells against activated hepatic stellate cells and increased the expression of NKG2D and tumor necrosis factor–related apoptosis-inducing ligand on liver natural killer cells. Blocking NKG2D or tumor necrosis factor–related apoptosis-inducing ligand with neutralizing antibodies markedly diminished the cytotoxicity of polyinosinic-polycytidylic acid–activated natural killer cells against activated hepatic stellate cells. *Conclusions:* Our findings suggest that natural killer cells kill activated hepatic stellate cells via retinoic acid early inducible 1/NKG2D-dependent and tumor necrosis factor–related apoptosis-inducing ligand–dependent mechanisms, thereby ameliorating liver fibrosis.

Viral hepatitis, which affects half a billion people worldwide, is a major cause of liver injury, fibrosis, and cirrhosis; however, the cellular and molecular mechanisms underlying the progression of liver disease during infection are not fully understood.[1,2] Emerging evidence suggests that natural killer (NK) cells, which are particularly enriched in the liver and activated by hepatitis viruses, play crucial roles in inducing antiviral immunity in the liver and inducing liver injury from elimination of virally infected hepatocytes.[3–8] NK cells are also involved in drug-induced hepatotoxicity[9,10] and in suppressing liver regeneration via an interferon (IFN)-γ–dependent mechanism[11]; however, the role of NK cells in the development and progression of liver fibrosis remains unknown.

Liver fibrosis is a common scarring response to chronic liver injury, regardless of etiology, including viral hep-

Abbreviations used in this paper: Ab, antibody; ASGM-1, anti–asialo GM-1; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FACS, fluorescence-activated cell sorting; FasL, Fas ligand; HSC, hepatic stellate cell; IFN, interferon; MNC, mononuclear cells; NK, natural killer; NKT, natural killer T cell; PDGF, platelet-derived growth factor; poly I:C, polyinosinic-polycytidylic acid; RAE1, retinoic acid early inducible 1; RT-PCR, reverse-transcription polymerase chain reaction; α-SMA, α-smooth muscle actin; TGF, transforming growth factor; TLR3, Toll-like receptor 3; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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atitis infection, alcohol abuse, nonalcoholic steatohepatitis, and autoimmune hepatitis.[12,13] Activation of hepatic stellate cells (HSCs) (formerly known as lipocytes, Ito cells, fat-storing cells, and perisinusoidal cells) is a key step in the development of liver fibrosis. Once activated, resident HSCs become fibrogenic myofibroblasts (activated HSCs), which express α-smooth muscle actin (α-SMA; a hallmark for activated HSCs) and produce large amounts of extracellular matrix proteins, such as collagen, resulting in liver fibrosis.[12,13] HSCs are activated by a variety of cytokines and growth factors, including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), tumor necrosis factor α, interleukin 1, angiotensin II, and leptin,[12,13] but are inhibited by antifibrogenic cytokines, such as interleukin 10, adiponectin, IFN-α/β, and IFN-γ.[12,13] For example, IFN-γ–deficient mice are more susceptible to liver fibrosis induced by carbon tetrachloride,[14] and the antifibrogenic effect of IFN-γ is believed to be mediated via inhibiting HSC activation and TGF-β signaling.[15–19] In this article, we show that NK cells act as antifibrogenic cells by killing activated HSCs. We also show that the cytotoxicity of NK cells is regulated by IFN-γ and the Toll-like receptor 3 (TLR3) ligand, polyinosinic-polycytidylic acid (poly I:C). Poly I:C is a potent stimulator for NK cells[20] and recognizes TLR3.[21]

At present, it is generally accepted that the cytotoxicity of NK cells against tumor cells and microbially infected autologous cells is determined by the balance between the effects of opposing NK cell receptors.[22–27] NK cells express several well-defined inhibitory receptors that recognize major histocompatibility complex class I molecules and inactivate NK cell functions.[22–26] These inhibitory receptors include inhibitory killer cell immunoglobulin-like receptor KIR, Ly-49A, and CD94/NKG2 receptors. Among the several stimulatory NK cell receptors, the NKG2D receptor is the best defined and is expressed on both human and mouse NK cells, where it is recognized by several ligands, including MHC class I chain–related gene A and UL16-binding protein for human NK cells and retinoic acid early inducible 1 (RAE1), histocompatibility 60, and mouse UL16-binding protein-like transcript 1 for mouse NK cells.[22–26] It has been shown that tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)$^+$ liver NK cells express low levels of the NK inhibitory receptor Ly-49A, which could be an important mechanism contributing to the cytotoxicity of liver NK cells against self hepatocytes[28] and tumors.[29] Here we show that NK cells kill activated HSCs, but not quiescent HSCs, which is likely mediated via an NKG2D/RAE1-dependent mechanism because expression of RAE1, the NKG2D ligand, was detected at high levels on activated HSCs but not on quiescent HSCs. Moreover, the cytotoxicity of NK cells against activated HSCs seems to be dependent on TRAIL also.

Materials and Methods

Materials

Poly I:C, Gey's balanced salt solution, and OptiPrep were purchased from Sigma (St Louis, MO). Pronase E and collagenase D were obtained from Roche (Indianapolis, IN).

Mice

Eight- to 10-week old male C57BL/6J, IFN-$\gamma^{-/-}$ mice (C57BL/6J background), perforin$^{-/-}$ mice (C57BL/6J background), and Fas ligand (FasL)$^{-/-}$ mice (C57BL/6Smn background) were purchased from the Jackson Laboratory (Bar Harbor, ME). CD1d$^{-/-}$ mice (natural killer T cell [NKT] deficient) on a BALB/c background were originally purchased from the Jackson Laboratory and backcrossed with C57BL/6J mice for at least 8 generations. All mice used in this study were housed in a specific pathogen–free facility and were cared for in accordance with National Institutes of Health guidelines.

Liver Injury and Fibrosis Induced by a 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine Diet and Carbon Tetrachloride

For 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet–induced liver injury, mice were fed a diet containing DDC (0.1% wt/wt in Purina 5015 mouse chow; Bio-Serv, Frenchtown, NJ) for 1–4 weeks. In the carbon tetrachloride–induced liver injury model, mice were injected intraperitoneally (IP) with carbon tetrachloride (10% in olive oil, 2 mL/kg, 3 times a week) for 2–3 weeks. After mice were killed, liver tissues were frozen in liquid nitrogen or fixed in 10% buffered formalin and embedded in paraffin.

Depletion of Natural Killer Cells by Anti–Asialo GM-1 Antibodies

To deplete NK cells (NK1.1$^+$CD3$^-$), anti-asialo GM-1 (ASGM-1) antibody (Ab; 100 μL per mouse; catalog no. 986-10001; Wako, Richmond, VA) was injected IP into mice. After 24 hours, depletion of NK (NK1.1$^+$CD3$^-$) cells was confirmed by flow cytometry.[11] To chronically deplete NK cells, mice were treated with anti–ASGM-1 every 48 hours for 2 weeks.

Treatment of Mice With Polyinosinic-Polycytidylic Acid

We have previously shown that injection of mice with poly I:C markedly induces accumulation and activation of NK cells in the liver, with the peak effect occurring at 24 hours and returning to basal levels at 48 hours after injection.[30] Therefore, for chronic treatment with poly I:C, mice were injected IP with poly I:C every 48 hours for 1–2 weeks. For mice in the

DDC diet and poly I:C cotreatment group, mice were injected with poly I:C 24 hours before DDC feeding and then maintained on the DDC diet with poly I:C injected every 48 hours for 1–2 weeks. For mice in the carbon tetrachloride and poly I:C cotreatment group, mice were injected IP with carbon tetrachloride (10% in olive oil, 2 mL/kg, 3 times a week) for 1 week and then treated with carbon tetrachloride (IP 3 times a week) and poly I:C (IP every 48 hours) for an additional 1–2 weeks. Control mice received carbon tetrachloride treatment plus saline injection. For acute poly I:C treatment, mice were injected IP with poly I:C once. After 16 hours, liver mononuclear cells (MNCs) or NK cells were isolated and used as effector cells in cytotoxicity assays or for reverse-transcription polymerase chain reaction (RT-PCR) analyses. The dose of poly I:C used in this study was 1 μg/g unless specified otherwise.

Histology and Immunohistochemistry

After routine processing, liver sections 5 μm thick were stained with H&E for histological analysis or Masson trichrome for collagen deposition. Immunostaining for α-SMA was performed on formalin-fixed, paraffin-embedded sections by using monoclonal α-SMA primary Ab (clone 1A4; Dako, Carpinteria, CA) and a biotinylated alkaline phosphatase–conjugated secondary Ab. Fast Red (Dako) was used as the chromagen/substrate. The collagen area/portal vein area and α-SMA–positive area/portal vein area were quantified by digital imaging with National Institutes of Health Scion Image and Adobe Photoshop (San Jose, CA) by using Masson trichrome and α-SMA–stained sections, respectively.

Double Staining With Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling and α-Smooth Muscle Actin Antibody

HSC apoptosis in the liver was determined by double staining with terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and α-SMA Ab, as described previously.[31] Apoptotic cells in sections of mouse livers were detected by using the In Situ Cell Death Detection Kit, POD (Roche, Indianapolis, IN), according to the manufacturer's instructions. TUNEL-positive nuclei were identified by the AEC Substrate Kit for Peroxidase from Vector Laboratories (Burlingame, CA) as red staining. Then, immunostaining for α-SMA with an alkaline phosphatase–conjugated secondary Ab was detected by a Vector Blue Alkaline Phosphatase Kit III. The number of TUNEL-positive (red) and α-SMA (blue) cells per field were counted.

Western Blotting

Western blotting was performed as described previously.[32] Protein bands were visualized by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

Reverse-Transcription Polymerase Chain Reaction

The RT-PCR was performed as described previously,[32] and the primers used are listed in Table 1.

Isolation and Culture of Hepatic Stellate Cells

HSCs were isolated from murine livers through sequential digestion with collagenase D (1 mg/mL) and pronase E (2 mg/mL) in Gey's balanced salt solution followed by discontinuous density centrifugation in 11.5% OptiPrep as described previously.[33,34] By use of trypan blue staining, the viability of the cells was determined to be 98%. The purity of the cells was also assessed visually by light microscopy examination of typical lipid droplet appearance, by fluorescence microscopy examination of vitamin A autofluorescence, and by immunochemistry examination of desmin expression. Typically, the purity of stellate cells was more than 90%.

Isolation of Mouse Liver Mononuclear Cells and Natural Killer ($NK1.1^+CD3^-$) Cells

Liver MNCs were isolated as described previously.[35] To purify liver NK cells ($NK1.1^+CD3^-$), $CD3^-$ liver MNCs were separated from liver MNCs by negative magnetic cell sorting according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Subsequently, $NK1.1^+CD3^-$ cells were purified by positive magnetic cell sorting by using anti-NK1.1 monoclonal Ab according to the manufacturer's protocol (Miltenyi Biotec). Approximately 93% of the MACS-purified cells were $NK1.1^+CD3^-$ cells.

Flow Cytometric Analysis of Natural Killer Cells, TRAIL, and NKG2D Expression

NK cells, T cells, NKG2D, Ly-49A, FasL, and TRAIL expression on MNCs from the livers of C57BL/6 mice and IFN-$\gamma^{-/-}$ mice were determined by using anti-NK1.1, anti-CD3, anti–Ly-49A, anti-FasL (BD PharMingen, San Diego, CA), and anti-TRAIL, anti-NKG2D Abs (eBioscience, San Diego, CA) by FACSCalibur (BD Biosciences, Mountain View, CA).

Cell-Mediated Cytotoxicity

The ToxiLight kit (Cambrex, Rockland, ME) was used to measure cell-mediated cytotoxicity against primary HSCs. Briefly, freshly isolated HSCs were plated onto round-bottom, 96-well plates. MNCs or NK cells were subsequently added as target cells at target–effector ratios ranging from 1:2 to 1:25. The release of adenylate kinase from damaged cells was measured from the culture medium by using the ToxiLight kit. To directly visualize the killing process and confirm whether MNCs or NK cells directly killed HSCs, the LIVE/DEAD Cell-Mediated Cytotoxicity Kit (Molecular Probes, Eugene, OR) was used. After target cells were loaded with a green fluorescent membrane stain, 3,3′-dioctadecyloxacarbocyanine, the plates were incubated with effector cells, and the coculture

Table 1. RT-PCR Primers Used in This Study

Genes	Forward (5′–3′)	Reverse (5′–3′)	PCR (base pairs)
NKG2D	GCA TTG ATT CGT GAT CGA AA	GCC ACA GTA GCC CTC TCT TG	374
Ly-49A	TTC TGG AAT CCC TCA ACA GG	GAA GGA ACC ACG AGC TGA AG	247
RAE1α	AGG TTG TGA GCT GCT CGA TT	GGG GTA GGA TCC TTG ATG GT	272
RAE1β	GCT GCA GTT CAA GAC ACC AA	GGG GTA GGA TCC TTG ATG GT	246
RAE1γ	ATT TGC ATT TGC GAT GTG AA	GCT GTA TTG CCT GGC ATT TT	382
RAE1δ	GCT GCA GTT CAA GCA ACC AA	CCA CGA AGC ACT TCA CTT CA	272
RAE1ε	GCT GCA GTT CAA GAC ACC AA	TCC ACT GAG CAC TTC ACG TC	285
H-60	TGT GCT GAT TTG TCC CAA AG	AAT TTG TTG AGG CAG CGA CT	299
Mult1	CAT GCC ATT GGT GCT CAT AG	TGC TTG TGT CAA CAC GGA AT	250
H2-D1	GGA AAA GGA GGG GAC TAT GC	GGA CAG GGT CCT GGT GTA GA	273
H2-D4	CCA GTG GAT GTA TGG CTG TG	TCT CCT TCC CTC CTG AGA CA	444
Clec2D	CTC GGT TTG ACA ACC AGG AT	GAT CCC GTT GTT GTT CAG GT	212
Perforin	GAT GTG AAC CCT AGG CCA GA	GGT TTT TGT ACC AGG CGA GA	155
TRAIL	CCC TGC TTG CAG GTT AAG AG	GGC CTA AGG TCT TTC CAT CC	220
HGF	TTC CCA GCT GGT CTA TGG TC	TGG TGC TGA CTG CAT TTC TC	237
TGF-β1	TTG CTT CAG CTC CAC AGA GA	TGG TTG TAG AGG GCA AGG AC	183
PDGFα	GAG ATA CCC CGG GAG TTG AT	AAA TGA CCG TCC TGG TCT TG	238
PDGFβ	CCT CGG CCT GTG ACT AGA AG	GGA CGA GGG GAA CAA CAT TA	267
TGF-α	CTG AAG GGA AGG ACT GCT TG	GTC CAC TGG CCT CTT CTC TG	272
FGF-2	AGC GGC TCT ACT GCA AGA AC	TCG TTT CAG TGC CAC ATA CC	299
IFN-γ	ACT GGC AAA AGG ATG GTG AC	TGA GCT CAT TGA ATG CTT GG	237
Fas L	TTG TTT TGG GGA GGA GTC AG	GGG GTA GGT GCT CAG TGT GT	175
JE	CCC AAT GAG TAG GCT GGA GA	GAA CTG CCT TTG CCT TCT TG	199
MIP-1β	CCC ACT TCC TGC TGT TTC TC	GAG GAG GCC TCT CCT GAA GT	236
I-TAC	ATG AAC GGC TGC GAC AAA GT	GCA TGT TCC AAG ACA GCA GA	221
α-SMA	CTG ACA GAG GCA CCA CTG AA	GAA GGA ATA GCC ACG CTC AG	288
TLR3	TTG TCT TCT GCA CGA ACC TG	CGC AAC GCA AGG ATT TTA TT	204
β-actin	AGC CAT GTA CGT AGC CAT CC	CTC TCA GCT GTG GTG GTG AA	227

Mult1, mouse UL16-binding protein-like transcript 1; H-60, histocompatibility 60; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; JE, monocyte chemoattractant protein-I; I-TAC, IFN-γ–inducible T-cell α chemoattractant.

was stained with propidium iodide. The results showed that most propidium iodide–stained cells were also positive for green fluorescence, thus indicating that stellate cells were killed by either MNCs or NK cells.

Statistical Analysis

Data are expressed as means ± SEM. To compare values obtained from 3 or more groups, 1-factor analysis of variance was used, followed by the Tukey post hoc test. To compare values obtained from 2 groups, the Student *t* test was performed. Statistical significance was taken at the $P < .05$ level.

Results

Polyinosinic-Polycytidylic Acid Treatment Attenuates Liver Fibrosis Induced by DDC Diet or Carbon Tetrachloride Injection

Feeding mice with a diet containing DDC induced liver injury as evidenced by elevation of serum alanine aminotransferase levels and necrosis in the liver (Figure 1*A*). Fibrosis was also induced, as shown by collagen deposition (blue staining by Masson trichrome) and HSC activation (α-SMA–positive staining; Figure 1*B* and *C*). To investigate the effect of NK cell activation on liver fibrosis, mice were fed the DDC diet and cotreated with poly I:C, which activates NK cells and induces accumulation of NK cells in the liver but produces only very mild liver injury.[30,36] Results show that poly I:C treatment did not significantly affect DDC diet–induced liver injury (Figure 1*A*), but it markedly reduced DDC-induced collagen deposition in the portal areas (Figure 1*B*) and HSC activation (α-SMA$^+$ cells; Figure 1*C*). The inhibitory effect of poly I:C on HSC activation was also confirmed by Western blot analyses. As shown in Figure 1*D*, poly I:C treatment prevented DDC diet–induced α-SMA protein expression in the liver.

These data show that treatment with poly I:C and DDC diet simultaneously prevented DDC-induced fibrosis and HSC activation. To determine whether the protective effect was due to the elimination of activated HSCs or the prevention of quiescent HSCs from becoming activated, mice were fed the DDC diet for 2 weeks to activate HSCs and then were maintained on the DDC diet and cotreated with poly I:C for 1 week. As shown in Figure 1*E*, treatment with poly I:C for 1 week markedly reduced liver fibrosis, as shown by reduced collagen

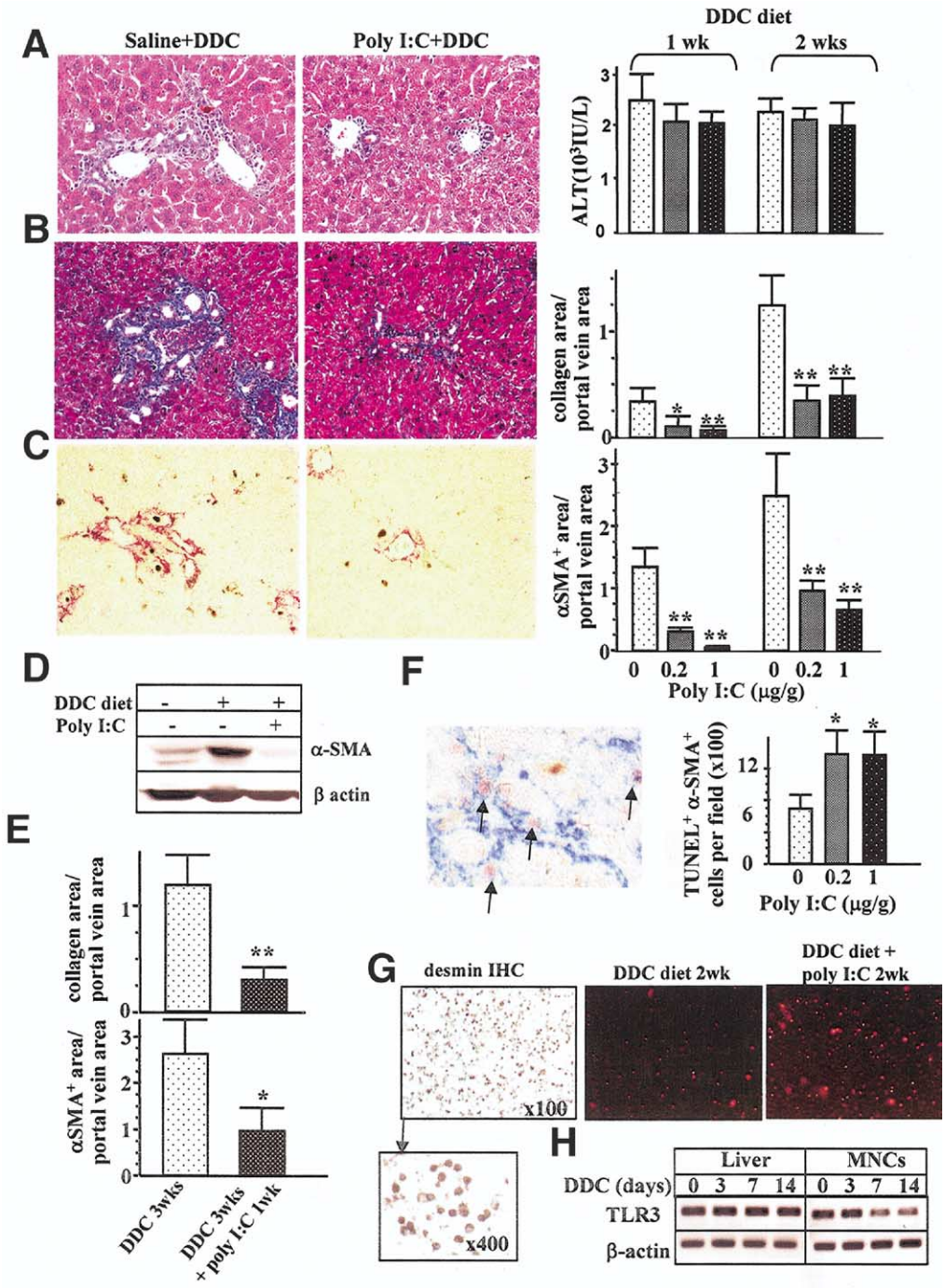

Figure 1. Poly I:C treatment attenuates liver fibrosis induced by the DDC diet. (*A–D*) Mice were fed the DDC diet and cotreated with or without poly I:C for 1–2 weeks as described in Materials and Methods. Mice were killed, and liver tissues were fixed and stained with H&E staining (*A*), Masson trichrome (*B*), or anti–α-SMA Ab (*C*). Serum levels of alanine aminotransferase are shown on the right side in (*A*). Collagen deposition and the α-SMA$^+$ area were quantified and shown on the right side in (*B*) and (*C*), respectively. Liver extracts from these mice were also subjected to Western blot analyses by using the anti–α-SMA Ab (*D*). (*E*) Mice were fed the DDC diet for 2 weeks to activate HSCs and were then maintained on the DDC diet and chronically treated with or without poly I:C (IP every 48 hours) for a 1-week period, followed by examination of collagen and α-SMA$^+$ cells in the liver. (*F* and *G*) Mice were fed the DDC diet and cotreated with or without poly I:C for 2 weeks as described in Materials and Methods. Mice were then killed, and liver tissues were stained with TUNEL and α-SMA to determine HSC apoptosis (*F*), or HSCs were isolated and stained with desmin and propidium iodide to determine the identity of HSCs and HSC death in vitro, respectively (*G*). A representative photograph of liver sections with double staining of TUNEL (red staining) and α-SMA (blue staining) from poly I:C + DDC–treated mice is shown in the left hand side of (*F*). (*H*) RT-PCR analyses of TLR3 and β-actin messenger RNA expression in the liver and liver MNCs from DDC-treated mice. Values in panels *A*, *B*, *C*, *E*, and *F* are shown as means ± SEM from 4–6 mice per each group. $^*P < .05$, $^{**}P < .01$ compared with the corresponding DDC group without poly I:C treatment. ALT, alanine aminotransferase; IHC, immunohistochemistry.

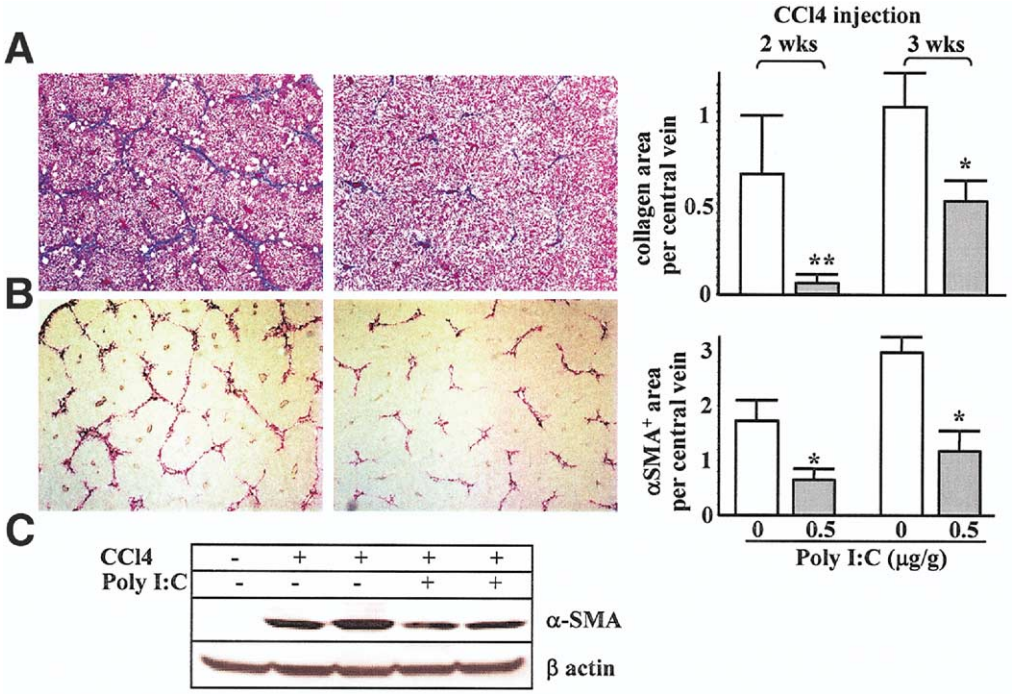

Figure 2. Poly I:C treatment attenuates liver fibrosis induced by carbon tetrachloride. Mice were injected with carbon tetrachloride and poly I:C for 2–3 weeks as described in Materials and Methods, followed by collection of liver tissues, which were fixed and stained with (*A*) Masson trichrome or (*B*) anti–α-SMA Ab. Collagen deposition and the α-SMA$^+$ area were quantified and are shown on the right side in panels *A* and *B*, respectively. Values in *A* and *B* are shown as means ± SEM from 3–5 mice per each group. $*P < .05$, $**P < .01$ compared with the corresponding DDC group without poly I:C treatment. (*C*) Liver tissues from these mice were subjected to Western blot analyses for α-SMA protein expression.

deposition and α-SMA activation in the portal area, thus suggesting that poly I:C treatment can reverse liver fibrosis. To examine whether poly I:C–induced reduction of liver fibrosis is due to the induction of stellate cell apoptosis, the TUNEL assay was performed. Results show that most of the TUNEL-positive cells were nonparenchymal cells located in the perisinusoidal region, and few hepatocytes were TUNEL positive, suggesting that hepatocyte death induced by DDC is necrosis. To further confirm whether the TUNEL-positive nonparenchymal cells were HSCs, double immunostaining with TUNEL and α-SMA was performed. As shown in Figure 1*F*, the number of TUNEL-positive and α-SMA–double-positive cells was significantly higher in mice from the poly I:C + DDC treatment group compared with the DDC + saline group (Figure 1*F*). In addition, we also isolated HSCs from saline + DDC–treated and poly I:C + DDC–treated mice, and these HSCs were then stained with propidium iodide to examine HSC apoptosis. As shown in Figure 1*G*, the purity of HSCs was identified by desmin positive staining; approximately 90% of the cells were desmin positive. Furthermore, the number of propidium iodide–stained HSCs was significantly greater in the poly I:C–treated group than the poly I:C–untreated group. These findings suggest that poly I:C treatment induces cell death in HSCs. In contrast, poly I:C treatment of control C57BL/6 mice receiving normal chow diet affected neither HSC activation nor HSC death (data not shown). Taken together, these data suggest that poly I:C treatment induces cell death in activated HSCs, but not in quiescent HSCs.

It has been reported that poly I:C is a potent stimulator for NK cells[20] and recognizes TLR3[21] and that poly I:C treatment up-regulates TLR3 expression on NK cells.[20] Next we wondered whether DDC feeding affects TLR3 expression in the liver. As shown in Figure 1*H*, expression of TLR3 in the liver remained unchanged but was down-regulated on liver MNCs after DDC feeding, thus suggesting that DDC feeding may decrease the response of liver MNCs to poly I:C treatment.

The effect of poly I:C on liver fibrosis was also examined by using another model of liver fibrosis induced by carbon tetrachloride. As shown in Figure 2, injection of carbon tetrachloride induced collagen deposition as detected by Masson trichrome staining (Figure 2*A*) and activation of HSCs as detected by α-SMA staining (Figure 2*B*). Poly I:C treatment markedly inhibited carbon tetrachloride–induced collagen deposition (Figure 2*A*) and α-SMA activation (Figure 2*B*). The latter was also confirmed by Western blot analyses. As shown in Figure

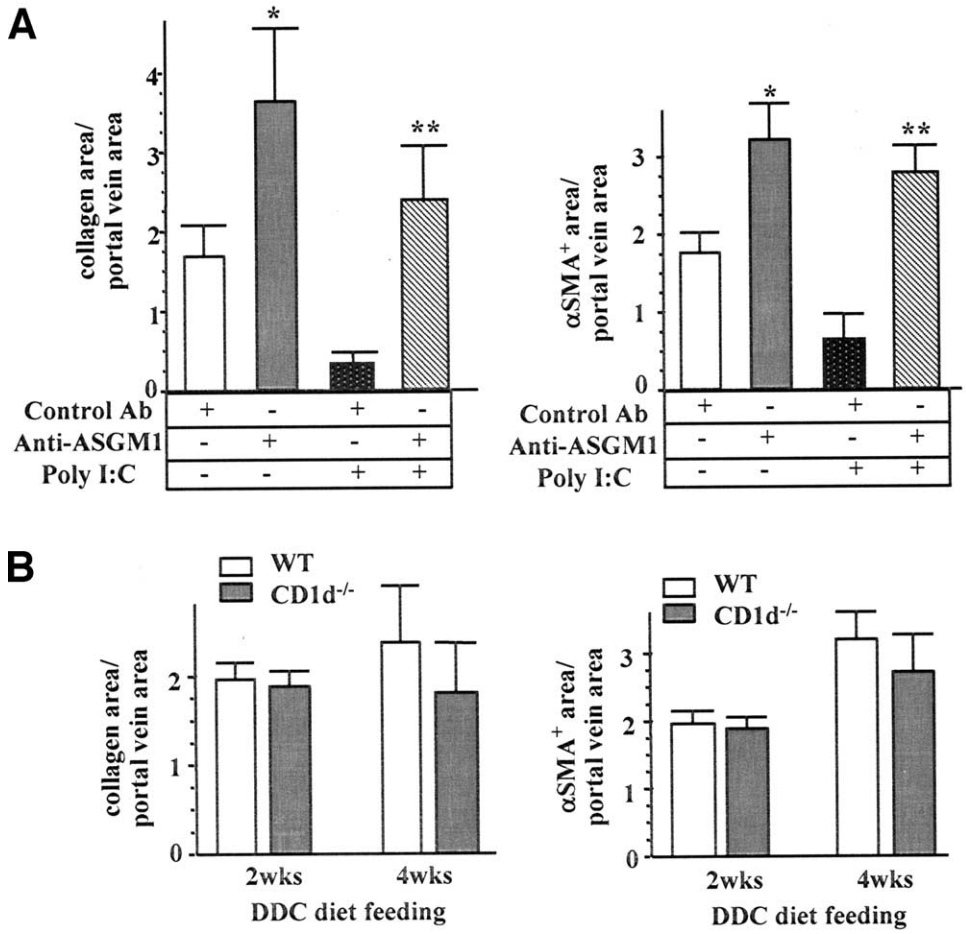

Figure 3. Effects of NK and NKT cells on liver fibrosis. (*A*) Depletion of NK cells enhances DDC-induced fibrosis and abolishes poly I:C inhibition of liver fibrosis. Mice were fed the DDC diet and chronically treated with control Ab or anti–ASGM-1 Ab and poly I:C, as described in Materials and Methods, for 2 weeks. Collagen and α-SMA$^+$ cells were stained and quantified. (*B*) Role of NKT cells in liver fibrosis. Wild-type mice and CD1d-deficient mice were fed the DDC diet for 2–4 weeks. Collagen and α-SMA$^+$ cells were stained and quantified. Values are shown as means ± SEM from 4 mice per group. $*P < .05$ compared with the corresponding control Ab-treated group, $**P < .01$ compared with the corresponding control Ab + poly I:C group. WT, wild type.

2*C*, carbon tetrachloride injection significantly induced α-SMA expression in the liver, which was inhibited by poly I:C cotreatment.

Depletion of Natural Killer Cells Enhances DDC-Induced Fibrosis and Abolishes Polyinosinic-Polycytidylic Acid Inhibition of Liver Fibrosis

The involvement of NK cells in poly I:C suppression of liver fibrosis was examined by using the anti–ASGM-1 Ab, which specifically depletes NK cells.[11] Depletion of NK cells slightly reduced DDC-induced liver injury (data not shown) but significantly enhanced DDC-induced fibrosis and elevation of α-SMA$^+$ cells (Figure 3*A*). Treatment with poly I:C suppressed DDC-induced fibrosis and elevation of α-SMA$^+$ cells in mice treated with control Ab, but not in mice treated with the anti–ASGM-1 Ab, thus suggesting that poly I:C suppression of liver fibrosis is mediated through an NK-dependent mechanism. In contrast, collagen deposition and α-SMA activation after DDC feeding were not different in CD1d-deficient mice compared with their controls (Figure 3*B*); this suggests that NKT cells play a minor role in liver fibrosis in this model.

Disruption of the Interferon γ Gene Enhances DDC Diet–Induced Fibrosis and Abolishes Polyinosinic-Polycytidylic Acid Suppression of Liver Fibrosis

IFN-γ–deficient mice were shown to be more susceptible to carbon tetrachloride–induced liver fibrosis.[14] This was also shown in this model of DDC-induced fibrosis, as shown in Figure 4, which shows that DDC-induced collagen deposition and elevation of α-SMA$^+$ cells were markedly enhanced in IFN-$\gamma^{-/-}$ mice compared with wild-type mice. TGF-β has

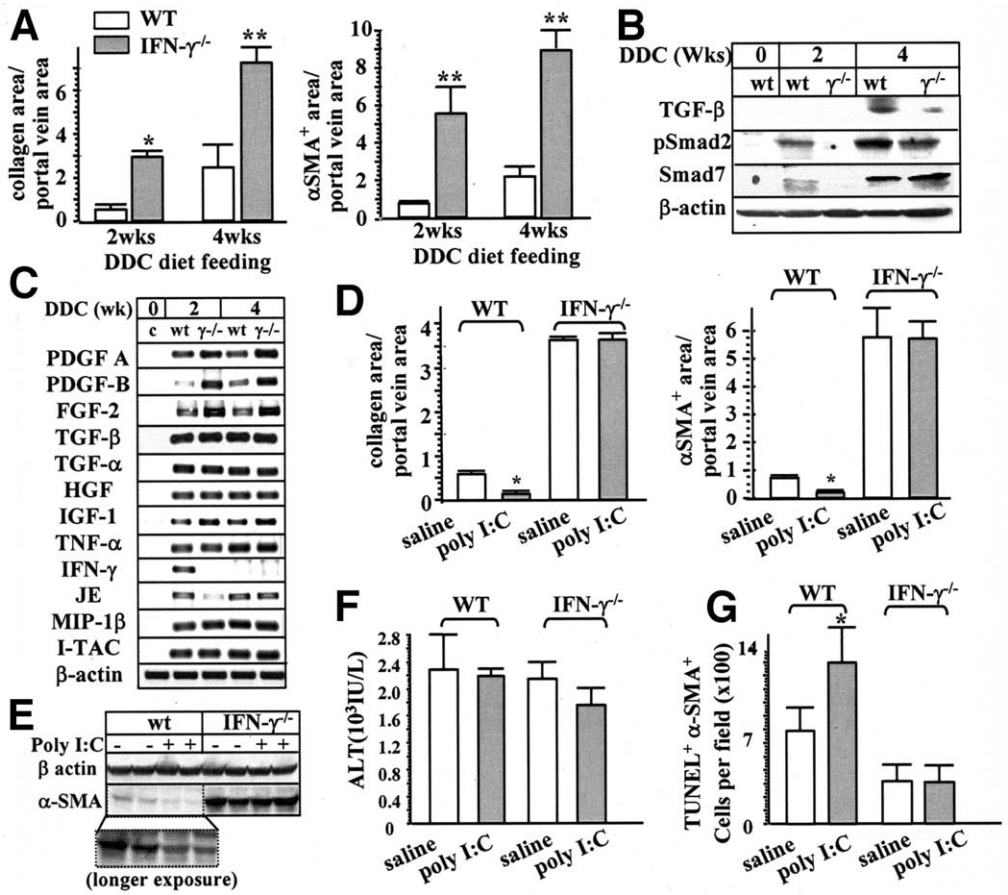

Figure 4. Disruption of the IFN-γ gene enhances DDC diet–induced fibrosis and abolishes poly I:C suppression of liver fibrosis. (*A–C*) Wild-type and IFN-$\gamma^{-/-}$ mice were fed the DDC diet for 2–4 weeks. Collagen and α-SMA$^+$ cells in the livers were stained and quantified (*A*). Liver tissues were also subjected to Western blot analyses with various Abs (*B*) and RT-PCR analyses using various primers, as indicated (*C*). (*D–G*) Wild-type and IFN-$\gamma^{-/-}$ mice were fed DDC and cotreated with or without poly I:C for 2 weeks as described in Materials and Methods. Collagen and α-SMA$^+$ cells in the livers were then stained and quantified (*D*); expression of α-SMA was analyzed by Western blot analysis (*E*), serum levels of alanine aminotransferase were measured (*F*), and HSC apoptosis was determined by double staining with TUNEL and α-SMA (*G*). Values are shown as means ± SEM from 4–6 mice per group. (*A*) $*P < .05$, $**P < .01$ compared with the corresponding wild-type group. (*D*) $*P < .05$ compared with the corresponding saline-treated wild-type group. WT/wt, wild type; C, wild type control; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; TNF, tumor necrosis factor; JE, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; I-TAC, IFN-γ–inducible T-cell α chemoattractant; ALT, alanine aminotransferase.

been shown to play an important role in liver fibrosis through activation of Smad2/3.[37–40] In contrast, Smad7, which is induced by IFN-γ,[19] acts as an antagonist for liver fibrosis.[41] In our study, immunohistochemistry analyses showed that expression of pSmad2 was reduced, whereas expression of Smad7 increased after DDC feeding in IFN-$\gamma^{-/-}$ mice compared with wild-type controls (data not shown). These results were further confirmed by Western blot analyses. As shown in Figure 4*B*, DDC feeding induced expression of TGF-β, pSmad2, and Smad7 in the liver. Expression of TGF-β and pSmad2 was reduced, but expression of Smad7 increased in IFN-$\gamma^{-/-}$ mice compared with wild-type mice after 4 weeks of DDC feeding. These findings suggest that the greater fibrosis severity in IFN-$\gamma^{-/-}$ mice was unlikely due to IFN-γ inhibition of TGF-β signaling. Moreover, the expression of several profibrotic factors, such as PDGF-A, PDGF-B, fibroblast growth factor 2, and insulin-like growth factor I, was greater in IFN-$\gamma^{-/-}$ mice than in wild-type controls after DDC feeding, which may contribute to accelerated fibrosis, as observed in IFN-$\gamma^{-/-}$ mice. Higher numbers of activated α-SMA$^+$ HSCs in IFN-$\gamma^{-/-}$ mice (Figure 4*A*) may account for the enhanced expression of profibrotic factors in these mice, because activated HSCs are the major source of these factors.[12,13]

To examine whether IFN-γ contributes to poly I:C suppression of liver fibrosis, we compared the effect of poly I:C on liver fibrosis in wild-type and IFN-$\gamma^{-/-}$ mice. As shown in Figure 4*D*, treatment with poly I:C attenuated DDC-induced fibrosis and HSC activation in

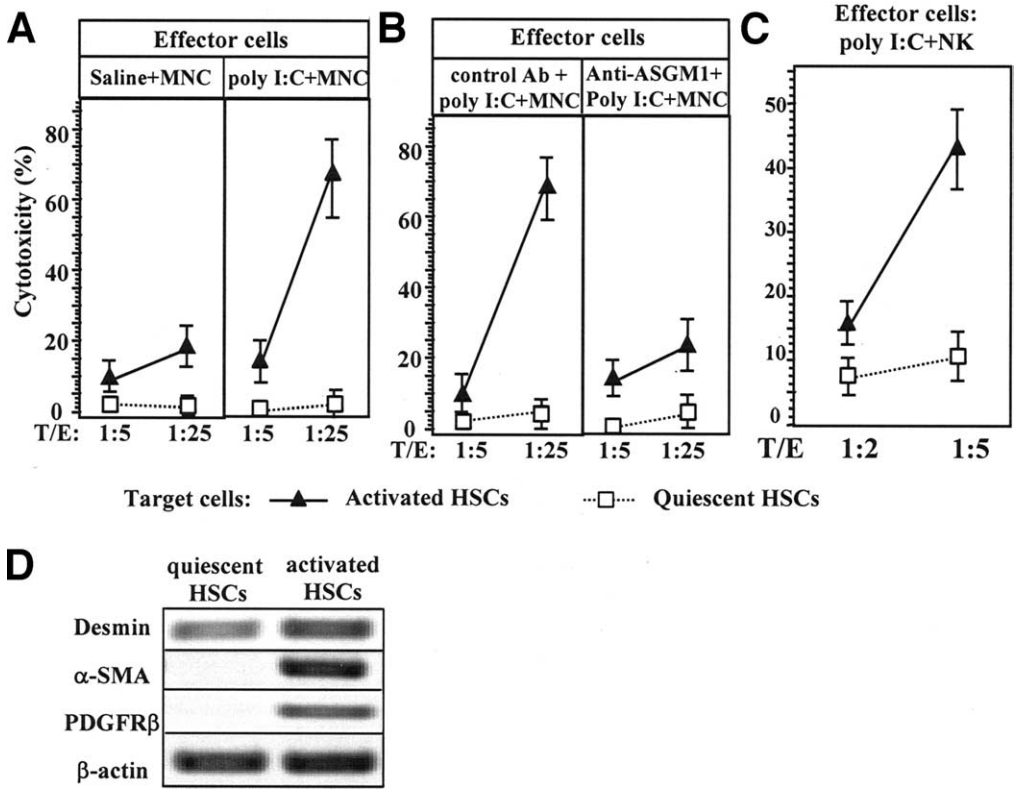

Figure 5. Activated HSCs are more susceptible to hepatic NK cell killing, which are enhanced by poly I:C treatment. Cell-mediated cytotoxicity assays were measured by using various target and effector cells. (*A*) MNCs isolated from mice acutely treated with saline or poly I:C as effector cells. (*B*) MNCs isolated from control Ab + poly I:C–treated mice and anti–ASGM-1 + poly I:C–treated mice were used as effector cells. (*C*) NK cells ($NK1.1^+CD3^-$) isolated from poly I:C–treated mice were used as effector cells. HSCs isolated from normal mice (quiescent HSCs) or 2-week DDC–fed mice (activated HSCs) were used as target cells in panels *A*, *B*, and *C*. (*D*) Western blot analyses of desmin, α-SMA, and PDGFRβ expression on quiescent and activated HSCs. T/E, target–effector ratio.

wild-type mice, but not in IFN-$\gamma^{-/-}$ mice, thus suggesting that poly I:C prevents fibrosis and HSC activation via an IFN-γ–dependent mechanism. This was further confirmed by Western blot analysis. As shown in Figure 4*E*, hepatic α-SMA expression was detected at much higher levels in DDC-fed IFN-$\gamma^{-/-}$ mice compared with DDC-fed wild-type mice. Cotreatment with poly I:C inhibited α-SMA expression induced by DDC feeding in wild-type mice, but not in IFN-$\gamma^{-/-}$ mice. Furthermore, serum levels of alanine aminotransferase after DDC feeding in IFN-$\gamma^{-/-}$ mice and wild-type mice were similar (Figure 4*F*). Finally, TUNEL analyses show that the number of TUNEL and α-SMA double-positive cells was significantly lower in DDC-fed IFN-γ–deficient mice compared with DDC-fed wild-type mice. Poly I:C treatment increased the number of apoptotic HSCs in wild-type mice but not in IFN-γ–deficient mice (Figure 4*G*). These findings suggest that the more severe liver fibrosis observed in IFN-$\gamma^{-/-}$ mice may be due to reduced HSC death but is not caused by enhanced liver injury in these mice.

Activated Hepatic Stellate Cells Are More Susceptible to Hepatic Natural Killer Cell Killing, Which Is Enhanced by Polyinosinic-Polycytidylic Acid Treatment

The above-mentioned data show that in vivo treatment with poly I:C affects activated HSCs, but not quiescent HSCs, via an NK-dependent mechanism. To further determine whether poly I:C–activated NK cells kill activated HSCs, in vitro cell-mediated cytotoxicity experiments were performed. As shown in Figure 5*A*, liver MNCs from saline-treated mice showed approximately 20% cytotoxicity against activated HSCs from DDC-fed mice, but not to quiescent HSCs from normal mice. Acute treatment with poly I:C markedly enhanced the cytotoxicity of liver MNCs against activated HSCs, whereas quiescent HSCs were resistant to such killing (*right panel* in Figure 5*A*). Next we examined whether NK cells were responsible for poly I:C–induced MNC killing against activated HSCs. First, Figure 5*B* shows that depletion of NK cells by the anti–ASGM-1 Ab abolished NK cell cytotoxicity against activated HSCs.

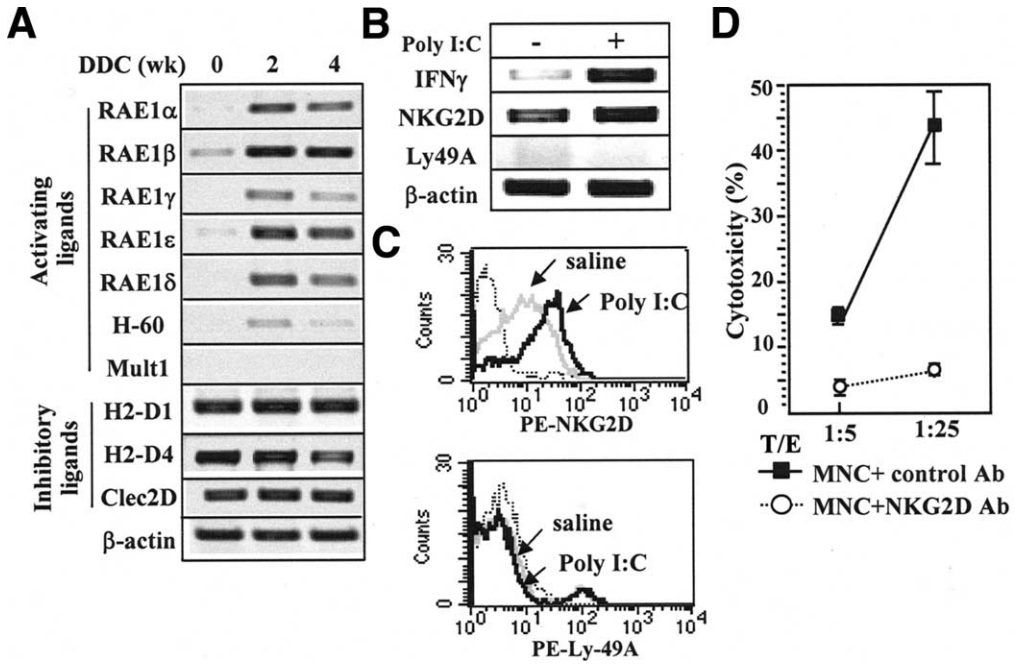

Figure 6. Evidence for the involvement of NKG2D/RAE1 in the cytotoxicity of liver MNCs against activated HSCs. (*A*) HSCs were isolated from DDC-fed mice or control mice. Expression of various NK receptor activating ligands and inhibitory ligands was detected by RT-PCR. (*B*) Liver MNCs were isolated from poly I:C–treated and untreated mice. Expression of IFN-γ, Ly-49A, and NKG2D on MNCs was detected by RT-PCR (*B*). (*C*) Liver MNCs from saline-treated and poly I:C–treated mice were analyzed by FACS with NK1.1, CD3, and NKG2D or Ly-49A Abs. Histograms for NKG2D or Ly-49A on NK ($NK1.1^+CD3^-$) cells from poly I:C–treated mice (*solid line*) are shown overlaid on the saline-treated group (*gray line*). *Dotted line* represents histograms for control Ab. (*D*) Liver MNCs isolated from poly I:C–treated mice were used as effector cells, which were incubated in vitro with control Ab or anti-NKG2D Ab. Activated HSCs from DDC-fed mice were used as target cells. Cytotoxicity was measured. Mult1, mouse UL16-binding protein-like transcript 1; H-60, histocompatibility 60; PE, phycoerythrin; T/E, target-effector ratio.

Second, purified NK cells ($NK1.1^+CD3^-$) from poly I:C–treated mice showed greater cytotoxicity against activated HSCs compared with MNCs from poly I:C–treated mice (note: the target–effector ratio used in purified NK killing was 1:2 and 1:5, respectively; Figure 5*C*). The identity of activated HSCs was confirmed by expression of α-SMA and PDGF-β, as shown in Figure 5*D*.

Evidence for the Involvement of RAE1/NKG2D in the Cytotoxicity of Liver Mononuclear Cells Against Activated Hepatic Stellate Cells

To understand why poly I:C–activated NK cells kill activated HSCs from DDC-fed mice, but not quiescent HSCs from normal mice, expression of NK receptor–activating ligands and inhibitory ligands present on HSCs was examined. As shown in Figure 6*A*, quiescent HSCs from normal C57BL mice expressed undetectable to very low levels of NK receptor–activating ligands. The expression of many of these ligands, including RAE1α, β, γ, ϵ, and δ, was markedly increased on activated HSCs from mice given the DDC diet for 2 and 4 weeks. Moreover, the expression of histocompatibility 60 was also slightly enhanced, whereas the expression of mouse UL16-binding protein-like transcript 1 was not induced. In contrast, quiescent HSCs expressed high levels of several NK receptor inhibitory ligands, most of which remained unchanged on activated HSCs after DDC feeding. Expression of H2-D4 on HSCs decreased slightly after DDC feeding (Figure 6*A*).

Next we examined the expression of the NK cell stimulatory receptor NKG2D and the inhibitory receptor Ly-49A on liver MNCs. As shown in Figure 6*B*, RT-PCR analyses showed that treatment of mice with poly I:C induced expression of IFN-γ and NKG2D on liver MNCs, whereas expression of Ly-49A was very low at baseline levels and was unaffected after poly I:C treatment. Consistent with these findings, fluorescence-activated cell sorting (FACS) analyses showed that liver NK cells expressed NKG2D, which increased after poly I:C treatment (Figure 6*C*). Expression of Ly-49A on NK cells was low and was not induced after poly I:C treatment (Figure 6*C*). Finally, treatment with NKG2D-neutralizing Abs abolished the cytotoxicity of poly I:C–treated liver MNCs against activated HSCs (Figure 6*D*), thus suggesting a

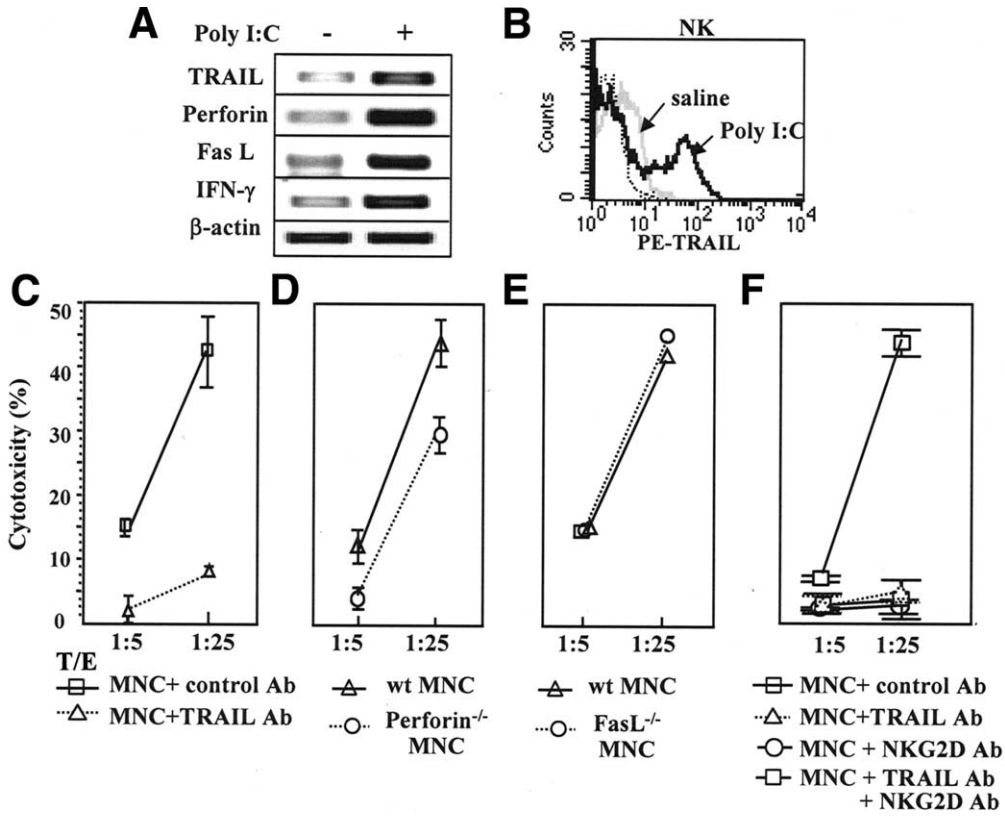

Figure 7. Hepatic NK cells kill activated HSCs via a TRAIL-dependent mechanism. (*A* and *B*) Hepatic MNCs were isolated from poly I:C–treated and untreated mice. Expression of various cytotoxic granule components and death receptor activators was detected by RT-PCR (*A*) and FACS analyses (*B*). In panel *B*, liver MNCs from saline-treated and poly I:C–treated mice were analyzed by FACS with NK1.1, CD3, and TRAIL Abs. Histograms for TRAIL on NK ($NK1.1^+CD3^-$) cells from poly I:C–treated mice (*solid line*) are shown overlaid on the saline-treated group (*gray line*). *Dotted line* represents histograms for control Ab. (*C*) Liver MNCs isolated from poly I:C–treated mice were used as effector cells, which were incubated with control Ab or anti-TRAIL Abs. (*D* and *E*) Liver MNCs isolated from poly I:C–treated wild-type or perforin$^{-/-}$ mice (*D*) or FasL$^{-/-}$ mice (*E*) were used as effector cells. (*F*) Liver MNCs isolated from poly I:C–treated mice were used as effector cells, which were incubated with either TRAIL Ab or NKG2D Ab or a combination of TRAIL and NKG2D Abs. Activated HSCs from DDC-fed mice were used as target cells in panels *C*–*F*. Cytotoxicity was measured. T/E, target-effector ratio; PE, phycoerythrin; wt, wild type.

critical role of NKG2D in the cytotoxicity of NK cells against activated HSCs.

Hepatic Natural Killer Cells Kill Activated Hepatic Stellate Cells Via a TRAIL-Dependent Mechanism

Expression of TRAIL, perforin, and FasL, which have all been implicated in the cytotoxicity of NK cells,[42] was examined. RT-PCR analyses showed that treatment with poly I:C increased expression of TRAIL, perforin, and FasL on liver MNCs (Figure 7*A*). FACS showed that liver NK cells expressed TRAIL, which was highly induced after poly I:C treatment (Figure 7*B*). Expression of FasL was mainly detected on NKT and T cells and induced after poly I:C treatment, whereas expression of FasL was undetectable on NK cells even after poly I:C treatment (data not shown). Incubation of MNCs with TRAIL neutralizing Abs markedly suppressed the cytotoxicity of poly I:C–treated liver MNCs against activated HSCs (45% in the control Ab–treated group vs 8% in anti-TRAIL Ab–treated group; Figure 7*C*). Liver MNCs from poly I:C–treated perforin$^{-/-}$ mice had slightly reduced cytotoxic activities against activated HSCs than liver MNCs from poly I:C–treated wild-type mice (45% in wild-type mice vs 32% in perforin$^{-/-}$ mice; Figure 7*D*), whereas the cytotoxicity of hepatic MNCs from poly I:C–treated wild-type and FasL$^{-/-}$ mice was similar (Figure 7*E*). These findings suggest that the cytotoxicity of poly I:C–treated hepatic MNCs against activated HSCs is predominantly mediated by a TRAIL-dependent mechanism, and perforin may only play a minor role, whereas FasL is not involved at all.

The results from Figures 6 and 7 suggest that both TRAIL and NKG2D contribute to the cytotoxicity of poly I:C–treated hepatic MNCs against activated HSCs. Next we examined whether TRAIL and NKG2D have a synergistic or additive effect. As shown in Figure 7*F*, treatment with either TRAIL Ab or NKG2D Ab markedly suppressed the cytotoxicity of poly I:C–treated

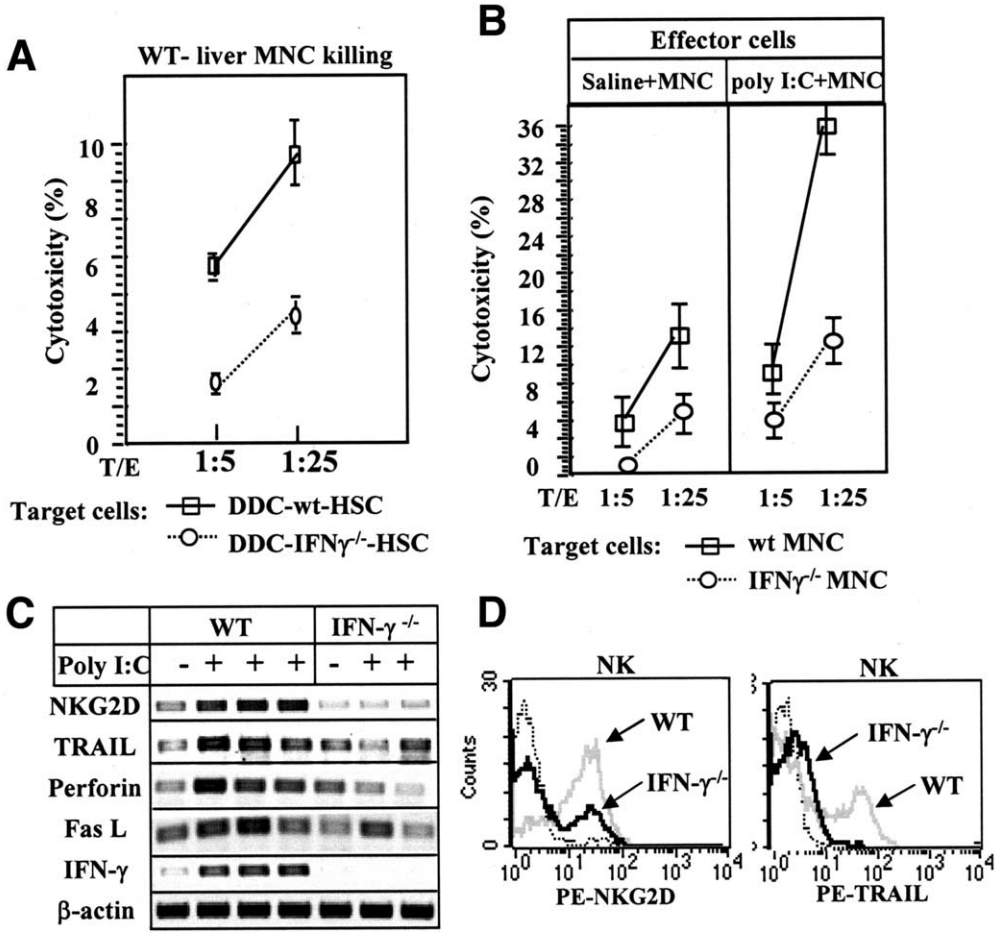

Figure 8. Hepatic MNCs from IFN-$\gamma^{-/-}$ mice produce less cytotoxicity against activated HSCs compared with wild-type mice. (*A*) Liver MNCs isolated from normal wild-type mice were used as effector cells. Activated HSCs from 2-week DDC–fed wild-type and IFN-$\gamma^{-/-}$ mice were used as target cells. Cytotoxicity was measured. (*B*) Liver MNCs isolated from saline-treated or poly I:C–treated wild-type or IFN-$\gamma^{-/-}$ mice were used as effector cells. Activated HSCs isolated from 2-week DDC–fed mice were used as target cells. Cytotoxicity was measured. (*C* and *D*) Expression of various genes on MNCs isolated from poly I:C–treated wild-type or IFN-$\gamma^{-/-}$ mice was determined by RT-PCR analyses (*C*) and FACS analyses (*D*). In panel *D*, liver MNCs from poly I:C–treated wild-type (WT/wt) mice and IFN-$\gamma^{-/-}$ mice were analyzed by FACS with NK1.1, CD3, and NKG2D or TRAIL Abs. Histograms for NKG2D or TRAIL on NK (NK1.1$^+$CD3$^-$) cells from poly I:C–treated IFN-$\gamma^{-/-}$ mice (*solid line*) are shown overlaid on the poly I:C–treated wild-type group (*gray line*). *Dotted line* represents histograms for control Ab. T/E, target–effector ratio; PE, phycoerythrin.

MNCs against activated HSCs, and the combination of TRAIL Ab and NKG2D Ab did not further inhibit it.

Hepatic Mononuclear Cells From Interferon $\gamma^{-/-}$ Mice Are Less Cytotoxic Against Activated Hepatic Stellate Cells Compared With Wild-Type Mice

To understand why IFN-$\gamma^{-/-}$ mice are more susceptible to DDC-induced fibrosis and why poly I:C prevention of DDC-induced fibrosis is abolished in IFN-$\gamma^{-/-}$ mice, we examined whether activated HSCs from DDC-fed IFN-$\gamma^{-/-}$ mice are resistant to liver MNC killing. As shown in Figure 8*A*, normal liver MNCs induced slightly greater cytotoxicity against activated HSCs from DDC-fed wild-type mice compared with DDC-fed IFN-$\gamma^{-/-}$ mice, whereas expression of NK receptor activating ligands (ie, RAE1α, β, γ, and ϵ) and inhibitory ligands (ie, H2-D1, H2-D4, and Clec2D) on activated HSCs were similar between these 2 groups (data not shown). These findings suggest that activated HSCs from IFN-$\gamma^{-/-}$ mice are slightly resistant to liver MNC killing compared with activated HSCs from wild-type mice and that this is unrelated to the differential expression of NK receptor ligands or inhibitory ligands. Next, the cytotoxicity of wild-type and IFN-$\gamma^{-/-}$ mouse liver MNCs against activated HSCs was examined. As shown in Figure 8*B*, compared with wild-type mice, hepatic MNCs from IFN-$\gamma^{-/-}$ mice showed lower cytotoxicity against activated HSCs. Poly I:C treatment significantly enhanced the cytotoxicity of wild-type mouse liver MNCs but only slightly enhanced the cytotoxicity of IFN-$\gamma^{-/-}$ mouse liver MNCs against activated HSCs,

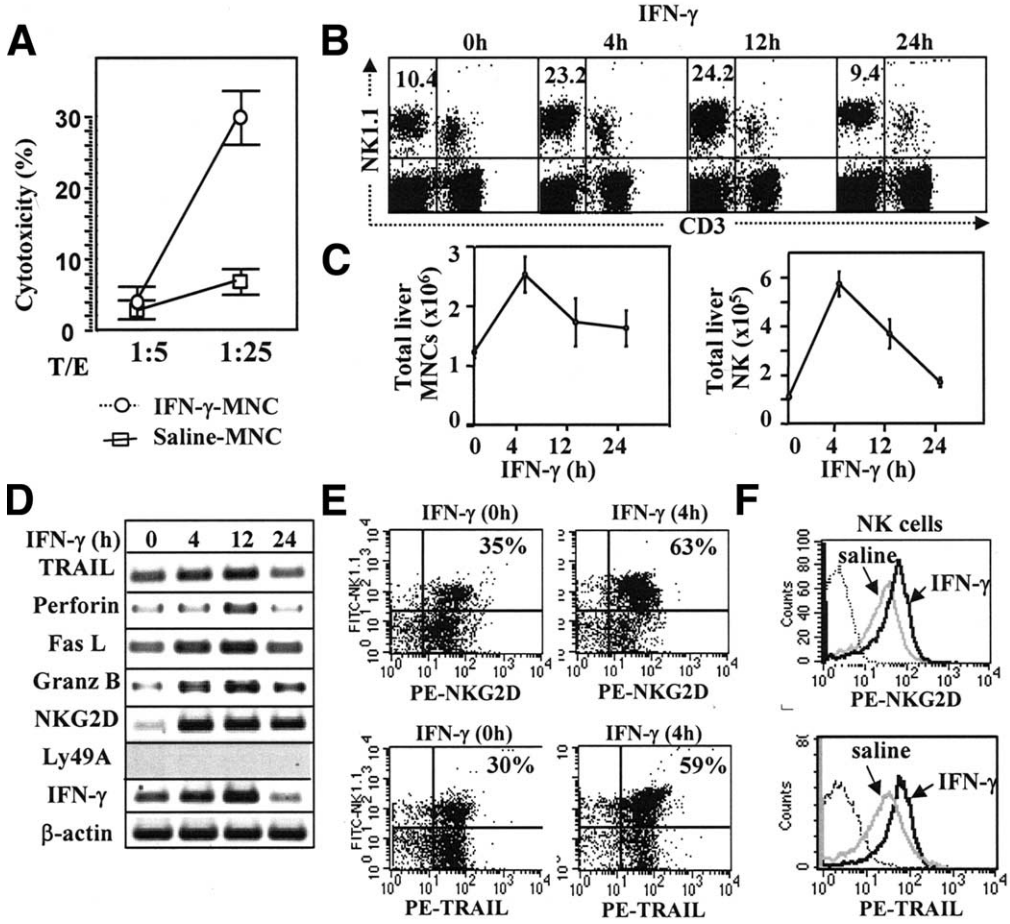

Figure 9. Treatment with IFN-γ activates NK cells in the liver and enhances the cytotoxicity of liver MNCs against activated HSCs. (*A*) C57BL/6 mice were treated with IFN-γ (0.5 μg/g intravenously) or saline for 4 hours. Liver MNCs were then isolated and used as effector cells. Activated HSCs isolated from mice fed DDC for 2 weeks were used as target cells. Cytotoxicity was measured. (*B–F*) C57BL/6 mice were treated with IFN-γ (0.5 μg/g intravenously) for various time points. Hepatic MNCs were isolated and analyzed by FACS with NK1.1 and CD3 Abs (*B*). The total number of hepatic MNCs and NK cells was counted (*C*). Hepatic MNCs were subjected to RT-PCR analyses by using various primers as indicated (*D*) and were analyzed by FACS with NK1.1 and NKG2D or TRAIL Abs (*E*) or with NK1.1, CD3, and NKG2D or TRAIL Abs (*F*). Histograms for NKG2D or TRAIL on NK ($NK1.1^+CD3^-$) cells from IFN-γ–treated mice (*solid line*) are shown overlaid on the saline-treated group (*gray line*). *Dotted line* represents histograms for control Ab. T/E, target-effector ratio; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

thus suggesting that IFN-γ contributes to the cytotoxicity of MNCs against activated HSCs.

To further explore the molecular mechanism underlying the involvement of IFN-γ in the poly I:C–induced cytotoxicity of NK cells against activated HSCs, we compared the expression of NKG2D, IFN-γ, and several cytotoxic granule components, as well as death receptor activators in wild-type and IFN-$\gamma^{-/-}$ mice after poly I:C treatment. As shown in Figure 8*C*, baseline levels of NKG2D expression were slightly lower in IFN-$\gamma^{-/-}$ mice compared with wild-type mice. Treatment with poly I:C induced expression of NKG2D in wild-type mice, but not in IFN-$\gamma^{-/-}$ mice. Similarly, poly I:C induction of TRAIL, perforin, FasL, and IFN-γ expression was also abolished in IFN-$\gamma^{-/-}$ mice compared with wild-type mice. FACS analyses confirmed that poly I:C induction of NKG2D and TRAIL expression on liver NK cells diminished in IFN-$\gamma^{-/-}$ mice compared with wild-type mice (Figure 8*D*).

Treatment With Interferon γ Activates Natural Killer Cells in the Liver and Enhances the Cytotoxicity of Liver Mononuclear Cells Against Activated Hepatic Stellate Cells

The above-mentioned data suggest that IFN-γ plays an essential role in poly I:C–mediated antifibrotic effects, which are mediated in part via direct inhibition of HSC proliferation and activation, as shown previously.[15–18] Next we wondered whether the antifibrotic effect of IFN-γ is also mediated via induction of NK cell cytotoxicity against activated HSCs. As shown in Figure 9*A*, IFN-γ treatment markedly enhanced the cytotoxicity of hepatic MNCs against activated HSCs. To under-

stand the underlying mechanism by which IFN-γ enhances hepatic MNCs killing activated HSCs, the effect of IFN-γ on hepatic NK cells was investigated. As shown in Figure 9*B* and *C*, treatment with IFN-γ induced accumulation of NK cells in the liver similar to poly I:C. Approximately 10% NK cells ($NK1.1^+CD3^-$) were detected in normal C57BL/6 mouse liver MNCs; the percentage of NK cells increased to 23%–24% after IFN-γ treatment. The peak effect occurred at 4 and 12 hours after injection and returned to a basal level at 24 hours. The total number of hepatic MNCs increased more than 2-fold, whereas the total number of hepatic NK cells increased approximately 6-fold at 4 hours after IFN-γ injection (Figure 9*C*). In contrast, IFN-γ treatment did not increase but rather decreased the percentage and total number of NK cells in the spleen (data not shown). Furthermore, Figure 9*D* shows that treatment with IFN-γ increased expression of TRAIL, FasL, granzyme B, NKG2D, and IFN-γ on liver MNCs. FACS analyses show that administration of IFN-γ into the mice increased the percentage of $NK1.1^+NKG2D^+$ cells from 35% at baseline to 63% at 4 hours after injection and increased the percentage of $NK1.1^+TRAIL^+$ from 30% at baseline to 59% at 4 hours after injection (Figure 9*E*). The density of NKG2D and TRAIL expression on NK ($NK1.1^+CD3^-$) cells was also increased after IFN-γ injection (Figure 9*F*). Taken together, these findings indicate that IFN-γ activates NK cells in the liver and enhances the cytotoxicity of liver MNCs against activated HSCs.

Discussion

In this article, we show (1) that NK cells are able to kill activated HSCs, central mediators in the development of liver fibrosis, thereby ameliorating liver fibrosis; (2) that NK cell cytotoxicity against activated HSCs is mediated via NKG2D/RAE1- and TRAIL-dependent mechanisms; and (3) that IFN-γ and the TLR3 ligand poly I:C inhibit liver fibrosis by enhancing NK cell cytotoxicity against activated HSCs.

Natural Killer Cells Kill Activated Hepatic Stellate Cells and Control Liver Fibrosis

Clinical data suggest that immunosuppression is an important permissive stimulus for progression of liver fibrosis. For example, progression to cirrhosis is accelerated in patients with hepatitis C virus (HCV) and human immunodeficiency virus coinfection or liver transplantation, who frequently require long-term immunosuppressive therapy,[43–45] thus suggesting that liver fibrosis is regulated by the host immune status. However, the mechanism by which the host immune status regulates liver fibrosis is poorly understood. Both T cells and Kupffer cells have been implicated in regulating liver fibrosis.[46,47] For example, $CD8^+$ T cells have been shown to promote liver fibrosis,[46] whereas Kupffer cells have been shown to play important but opposing roles in liver fibrosis through favoring extracellular matrix accumulation during ongoing injury but enhancing matrix degradation during recovery.[47,48] It was also reported that HSCs can be killed by activated Kupffer cells[49] but not by T cells,[50] which may be another mechanism involved in controlling liver fibrosis by immune cells. However, the role of NK cells in liver fibrosis remains obscure, although hepatic NK cells are activated after viral infection.[3–5,51–54] Treatment with anti-NK1.1 Abs, which deplete both NK ($NK1.1^+CD3^-$) and NKT ($NK1.1^+CD3^+$) cells, enhanced *Schistosoma mansoni*–induced liver fibrosis,[55] thus suggesting that NK and/or NKT cells may be involved in negative regulation of liver fibrosis. Here we provide several lines of evidence suggesting that NK cells ameliorate liver fibrosis via killing activated HSCs in addition to releasing IFN-γ. First, treatment with poly I:C activates NK cells and induces accumulation of NK cells in the liver,[30,36] resulting in attenuation of DDC-induced liver fibrosis induced by DDC or carbon tetrachloride. Second, depletion of NK cells ($NK1.1^+CD3^-$) enhanced liver fibrosis and abolished poly I:C suppression of liver fibrosis. Third, treatment with poly I:C induced activated HSC apoptosis in DDC-fed mice (Figure 1), which is likely due to the induction of activated HSC death and not to quiescent HSC death, because in vivo poly I:C treatment reduced the number of activated HSCs in DDC-fed mice (Figure 1), whereas the same treatment left the number of quiescent HSCs unchanged in mice fed normal chow (data not shown). Finally, consistent with the in vivo data, hepatic NK cells produced significantly greater cytotoxicity against activated HSCs than quiescent HSCs (Figure 5). Taken together, our findings suggest that activated NK cells suppress liver fibrosis via killing activated HSCs.

Natural Killer Cells Kill Activated Hepatic Stellate Cells Via NKG2D- and TRAIL-Dependent Mechanisms

Emerging evidence suggests that the cytotoxicity of NK cells is determined by the balance between activation of stimulatory and inhibitory receptors on NK cells.[22–26] The interaction of the stimulatory receptor NKG2D and its ligand RAE1 has been extensively investigated and implicated in the cytotoxicity of NK cells.[22–26] RAE1 was originally identified as a retinoic

acid–induced early gene in the embryonic carcinoma F9 cell line[56] and was later identified as a ligand for stimulatory NKG2D receptors.[57] Expressed at high levels in the embryonic mouse cell line and many other tumor cell lines, RAE1 is found at very low levels in tissues from adult mice.[57] Expression of RAE1 was also detected on nonneoplastic cells such as dorsal root ganglia neurons[58] and lipopolysaccharide-activated or poly I:C–activated macrophages[59]: this is likely involved in the cytotoxicity of NK cells against these cells. Here we show that expression of RAE1α, β, γ, δ, and ϵ was barely detectable on quiescent HSCs from mice given normal chow but was detected at high levels on activated HSCs from DDC-fed mice (Figure 6), which correlate with the resistance and susceptibility of quiescent HSCs and activated HSCs to NK cell killing, respectively (Figure 5). The activity of RAE1 is currently believed to be mediated through binding to NKG2D receptors on NK cells and the subsequent activation of NK cells.[22–25] The interaction between RAE1 and NKG2D may also be an important mechanism contributing to the cytotoxicity of NK cells against activated HSCs because treatment with NKG2D-neutralizing Abs abolished NK cell–mediated lysis of activated HSCs (Figure 6).

Several cytotoxic granule components, such as perforin and granzymes and the death receptor activators TRAIL and FasL, have also been implicated as important effectors in NK cell killing.[42] It has been reported that liver NK cells express TRAIL, which plays an important role in surveillance of tumor metastasis.[29,60] In this study, we show that poly I:C treatment increased expression of TRAIL on liver NK cells, and blocking TRAIL with neutralizing Abs significantly suppressed NK cell cytotoxicity against activated HSCs (Figure 7), thus indicating that TRAIL is an important mediator involved in NK cell lysis of activated HSCs. Although perforin has been implicated in NK cell killing of tumor cells,[42,61,62] perforin may play only a minor role in the cytotoxicity of NK cells against activated HSCs because NK cell cytotoxicity against activated HSCs was reduced slightly in perforin$^{-/-}$ mice compared with wild-type mice (Figure 7*D*). Furthermore, FACS analyses show that Fas L was detected on NKT and T cells, but not on NK cells (data not shown), which is consistent with a previous study[28] and suggests that FasL may not be involved in NK cell cytotoxicity against activated HSCs. Indeed, the cytotoxicity of liver MNCs against activated HSCs was not different in FasL$^{-/-}$ mice compared with wild-type mice (Figure 7*E*). Collectively, our findings suggest that both NKG2D and TRAIL contribute to NK cell cytotoxicity against activated HSCs, whereas perforin and Fas L play minor roles. The findings that coadministration of NKG2D and TRAIL Abs did not further reduce the NK cell cytotoxicity (Figure 7*F*) suggest that NKG2D and TRAIL do not additively or synergistically activate NK cell killing and that they may sequentially stimulate NK cell cytotoxicity against activated HSCs. It is likely that RAE1 proteins on activated HSCs bind NKG2D on NK cells and subsequently activate NK cells to release TRAIL to kill activated HSCs.

Although we clearly show that NK cells can kill activated HSCs, the effect of activated HSCs on NK cell activity is unclear. It is known that activated HSCs are the major source for TGF-β production during the fibrogenic response[37,63] and that TGF-β inhibits NK cell activity.[64,65] Therefore, it is plausible that activated HSCs inhibit NK cell activity through releasing TGF-β, which may account in part for the decreased NK cell activity in cirrhotic patients.[66] Further studies are needed to confirm this hypothesis.

Polyinosinic-Polycytidylic Acid, a Toll-Like Receptor 3 Ligand, and Interferon γ Inhibit Liver Fibrosis by Enhancing the Cytotoxicity of Natural Killer Cells Against Activated Hepatic Stellate Cells

The antifibrogenic role of IFN-γ is believed to be mediated via inhibition of HSC activation or inhibition of TGF-β signaling.[15–19] Here we provide evidence suggesting that enhancement of the cytotoxicity of NK cells against activated HSCs may be another important mechanism contributing to TLR3 ligand/IFN-γ amelioration of liver fibrosis. Poly I:C treatment enhances the cytotoxicity of NK cells against activated HSCs, which is dependent on IFN-γ because disruption of the IFN-γ gene abolishes poly I:C induction of NK cell cytotoxicity against activated HSCs (Figure 8*B*). Furthermore, RT-PCR and FACS analyses show that poly I:C treatment induces expression of NKG2D and TRAIL on NK cells from wild-type mice but not from IFN-γ$^{-/-}$ mice (Figure 8*C*), which correlates with diminished cytotoxicity of liver MNCs from poly I:C–treated IFN-γ$^{-/-}$ mice against activated HSCs (Figure 8*B*). In contrast, expression of NK inhibitory receptor ligands (such as RAE1 and H2-D) on activated HSCs was not regulated by IFN-γ, because similar levels were observed between wild-type and IFN-γ$^{-/-}$ mice (data not shown). Finally, administration of IFN-γ induced accumulation of NK cells in the liver, enhanced expression of both TRAIL and NKG2D on liver MNCs, and enhanced the cytotoxicity of NK cells against activated HSCs (Figure 9). Taken together, these findings suggest that poly I:C enhances the cytotoxicity of liver MNCs against activated HSCs via an IFN-γ–dependent induction of NKG2D and

TRAIL. This likely contributes to the antifibrogenic effect of poly I:C/IFN-γ on liver fibrosis in conjunction with IFN-γ direct inhibition of HSC proliferation and activation.[15–18]

Conclusions

In our investigation, we show in a mouse model of DDC-induced fibrosis that NK cells kill activated HSCs via RAE1/NKG2D- and TRAIL-dependent mechanisms, thereby controlling liver fibrosis. Clinical evidence suggests that HCV-infected patients with suppressed immune functions and low NK cell activity have accelerated progression of liver fibrosis.[43–45] For example, progression of liver fibrosis is much faster in HCV patients who are coinfected with human immunodeficiency virus[44] or who have received liver transplants (required long-term treatment with immunosuppressive drugs) or alcohol abuse (suppression of immune functions).[43–45] Treatment with IFN-α or IFN-γ, both of which are potent activators of NK cells,[67,68] prevents and ameliorates liver fibrosis in HCV patients.[69,70] These data suggest that NK cells may play important roles in the suppression of liver fibrosis in humans. Decreased NK cell cytotoxicity was reported in chronic HCV–infected patients,[71] which may contribute to accelerated fibrosis in these patients. It has been well documented that alcohol drinking inhibits NK cell activity,[72,73] which may be an important mechanism contributing to rapid progression of liver fibrosis in alcoholics with HCV infection.[74–76] Therefore, stimulation of NK cell activity, such as IFN-α and IFN-γ therapy in viral hepatitis patients, may have beneficial effects not only in clearing the viruses, but also in suppressing liver fibrosis.

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